Journal of Medical Virology 52:50–60 (1997)

Comparative Study of Poliovirus Excretion After Vaccination of Infants With Two Oral Polio Vaccines Prepared on Vero Cells or on Primary Monkey Kidney Cells

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A comparative study was designed to assess the bioequivalence of 2 oral poliovaccines (OPV) produced on 2 different cell systems: primary monkey kidney (PMK) cells and the Vero cell line. The Vero cell line has been used to overcome the problem of obtaining a regular supply of high quality monkeys that are devoid of latent viruses. For this study, 9 children were vaccinated with PMK-OPV and 12 children with Vero-OPV. The comparison covered poliovirus excretion, reversion of polioviruses in the 5′-noncoding region, and immunogenicity. Major molecular markers in the 5′-noncoding region related to neurovirulence already had been identified at position 480 for type 1, position 481 for type 2, and position 472 for type 3 poliovirus. Two nucleic-acid based methods were designed for studying these positions: a RT-PCR followed by sequencing, which required preliminary culture and cloning; and a type-specific nested PCR followed by sequencing, which enabled direct detection and genotyping of polioviruses. Twenty-eight stool specimens were analyzed by this second method with no PCR inhibition problem.

The use of Vero cell line did not modify the global pattern of poliovirus excretion, reversion frequency, or seroconversion. These results provide additional support for the use of the well-characterized Vero cell line in OPV manufacturing. ***J. Med. Virol. 52:50–60, 1997.***

KEY WORDS: poliovirus; reversion; neurovirulence; nested PCR, stool; direct detection

INTRODUCTION

The number of reported poliomyelitis cases has decreased drastically since the introduction of two vaccines: inactivated poliovaccine (IPV) [Salk and Salk, 1977], and live attenuated oral poliovaccine (OPV) [Sabin and Boulger, 1973]. OPV, traditionally prepared on primary monkey kidney (PMK) cells, is widely used and reproduces the mechanism of natural infection. Polioviruses, which multiply in the intestinal tract and are fecally excreted, may undergo the reversions sometimes responsible for vaccine associated paralytic poliomyelitis (VAPP) cases. For example, in the United States from 1973 to 1984, there were 105 such cases (i.e., 76% of the 138 total reported cases of poliomyelitis). Most cases occurred after administration of the first OPV dose. The contacts of OPV recipients were mainly affected [Nkowane et al., 1987].

The poliovirus genome consists of a single strand of positive sense RNA of approximately 7,500 nucleotides. The viral RNA encodes a single, long open reading frame that is translated into a polyprotein with a molecular weight of approximately 250,000. This protein is processed by 2 types of virus-encoded proteases to produce functional viral polypeptides. The viral RNA contains a 5′-noncoding region of approximately 743 nucleotides, which is covalently linked to a small protein VPg, and a 3′-noncoding region of approximately 73 nucleotides with a polyadenylated tail.

A neurovirulence-related, major molecular marker is located in the 5′-noncoding region for each poliovirus serotype: for serotype 1, it is located at nucleotide 480; for serotype 2, at nucleotide 481; and for serotype 3, at

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Accepted 28 November 1996

nucleotide 472 [Minor, 1992]. Back mutation of this nucleotide among OPV recipients has been observed already in both healthy vaccinees [Evans et al., 1985; Minor and Dunn, 1988; Tatem et al., 1991] and from VAPP-derived isolates [Otelea et al., 1993; Pollard et al., 1989; Equestre et al., 1991; Cann et al., 1984; Evans et al., 1985]. Intertypic recombinant strains having serotype 2 or 3 specificity also were isolated from healthy vaccinees [Kew and Nottay, 1984; Minor et al., 1986; Cammack et al., 1988], as well as among VAPP patients [Macadam et al., 1989; Lipskaya et al., 1991; Furione et al., 1993]. However, the enhancement of neurovirulence was not clearly related to such intertypic recombination [Macadam et al., 1989].

The availability of primary monkey kidney cells devoid of latent viruses for vaccine production has been strictly dependent upon a regular supply of healthy monkeys. It was decided to prepare the OPV on a validated working cell bank of Vero cells to overcome supply problems [Montagnon, 1989].

The aim of this pilot small-scale study was to compare poliovirus excretion, reversion patterns, and immunogenicity of two kinds of OPV, one produced on PMK cells (PMK-OPV), and the other on Vero cells (Vero-OPV).

The study was conducted using classic virological methods (e.g., infectivity assay, neutralization assay, and phenotypic characterization) and the more recently introduced nucleic acid-based technologies for genotypic characterization which enable each group of analytical methods to be evaluated.

MATERIALS AND METHODS

Design of the Study

The study was conducted during 1987 and 1988 in 2 French nurseries (A and B) of equal size and socioeconomic origins of the population of children. Children (aged 6–8 months and not previously immunized against polio) were given, on days 0, 30, and 60, one dose of OPV prepared either on Vero or on PMK cells and standardized according to the European Pharmacopoeia. Defined exclusion criteria included immune deficiency, repeated infectious diseases, gamma globulin injection 6 weeks before or 2 weeks after vaccination, or OPV vaccination of a contact during the study.

In nursery A, 12 children were given Vero-OPV. They were matched with 17 contacts. In nursery B, 9 children were given PMK-OPV and likewise were matched with 12 contacts.

Blood samples were collected from vaccinees on days 0, 60, and 90, and stools were collected on days 0, 1, 2, 3, 4, 6, 10, 20, and 30 after each vaccine feeding and on days 100, 110, and 120.

For each vaccinee, contacts were chosen to search for evidence of virus transmission. The average age of these contacts was 19 months, and most had been previously immunized with IPV. Stools were collected from these contacts on day 0, and at subsequent 10-day intervals until day 90.

Virologic Methods

Tissue culture. Hep 2 and Vero cells were provided either by the National Reference Center for Enteroviruses (Lyon, France) or by Pasteur Mérieux Sérums et Vaccins (P.M.sv.; Lyon, France).

Cell lines were propagated in Eagle minimal essential medium (MEM) supplemented with 7% fetal calf serum, 1% amino acids, glutamin, and antibiotics.

Primary isolation of poliovirus strains and cloning. A 10% (weight/volume) stool suspension was prepared in Eagle MEM supplemented with glutamin and antibiotics. This initial suspension was labelled "P0" stool and registered with a code number. "P0" stools were inoculated onto Vero and Hep 2 cells and incubated at 36.5°C for virus recovery. The procedure described by Dulbecco and Vogt [1954] was adopted for cloning the excreted polioviruses. Viral strains were isolated from stools collected 6 days after the first and the second OPV doses (on day 6 and day 36). They were cloned on 6-well culture plates. Briefly, after neutralization of the primary viral isolate with a pool of antisera (2 h at 35°C), the virus/antiserum mixture was adsorbed to Hep 2 cells (1 hr at 35°C) and removed. Culture plates were overlaid with 1.5% agar in MEM supplemented with 1% fetal calf serum and incubated at 35°C under 5% CO_2 for 4 days. Two successive plaque purifications were performed to select the clones.

Infectivity assays. Titers of viral strains were determined on Vero cells at 36.5°C in microtitration plates and expressed as 50% cell culture infectious doses ($CCID_{50}$) of the virus per 50 μl in accordance with the Reed and Muench method [Reed and Muench, 1938].

Identification and intratypic differentiation of polioviruses. Poliovirus identification was performed on Vero cells at 36.5°C by using neutralization with polyclonal antisera. Intratypic differentiation was done using type and strain specific monoclonal antibodies provided by Institut Pasteur (Paris, France) [Crainic et al., 1983].

All polioviruses were characterized for phenotypic markers (replicative capacity at temperature = rct40) by incubation at 36.5°C, 39.2°C, and 40.0°C or 40.3°C to determine themoresistance [WHO, 1990].

Titration of neutralizing antibodies. Neutralizing antibody assays were done with a micromethod that used Vero cells. Sera were serialy twofold diluted from 1/5 to 1/2560. Equal volumes of diluted serum containing 100 $CCID_{50}$ of the challenge virus were incubated (3 hr at 36.5°C) before the cell suspension was added. Culture plates were then incubated at 37°C with 5% CO_2 for 5 days. Titers were expressed as the highest dilution of serum in a volume of 50 μl that inhibited the cytopathic effect of 100 $CCID_{50}$.

Neurovirulence test on monkeys. One type 1, one type 2, and two type 3 cloned isolates (i.e., excreted polioviruses found to be reverted at the studied positions) were submitted for neurovirulence testing in

monkeys. Viruses were assayed by intraspinal inoculation of either cynomolgus or rhesus macaques using the standard World Health Organization approved test [WHO, 1990].

Genetic Studies

Reference strains. OPV batches of Sabin type 1, type 2, and type 3 and inactivated poliovaccine batches (Mahoney, MEF-1, and Saukett) were vaccine batches produced on Vero cells by P.M.sv. The appropriate reference wild strains (Brunhilde, Lansing and Leon) were obtained from American Type Culture Collection.

Stool samples. Twenty eight "P0" stool samples collected at day 6 and 36 were provided by 14 vaccinated children randomly selected (i.e., 7 PMK-OPV and 7 Vero-OPV). They were used for direct detection and genotyping of poliovirus by type-specific nested-PCR followed by sequencing.

Cloned isolates. Eighteen vaccinated children (10 with Vero-OPV and 8 with PMK-OPV) were studied. Twenty-two stool samples collected at day 6 and/or 36 were randomly selected. They generated a large number of clones, from among which 70 were randomly selected. These 70 clones included 2 clones per isolated poliovirus type for each stool. These cloned isolates were used for phenotypic characterization and genotypic characterization by RT/PCR followed by sequencing.

Viral lysis. The initial sample volumes were 2 μl of 1/10 dilution for Sabin strains, 5 μl of neat sample for all other strains, 5 μl for cloned isolates, and 5 μl for "P0" stools.

Five μl of NET buffer (100 mM NaCl, 10 mM EDTA, 10 mM Tris, pH = 8.0) was added to 20 U of human placental ribonuclease inhibitor (RNase Inh; Boehringer Mannheim; Meylan, France) and mixed with the sample. The final volume was adjusted to 10.5 μl with water for injection (P.M.sv.), and the mixture was placed in boiling water for 5 min. cDNA synthesis was performed immediately after.

RT/PCR and sequencing of the cloned isolates.

Primers. The primers Syl1-P, Lau2-P, RB1, and RB2 were specific for locations in the 5′-noncoding region. These were selected for their ability to detect all strains of poliovirus.

A first PCR primer set (Syl1-P and RB2) was used to obtain a 184–185 bp PCR product. (The fragment size depended on poliovirus type). Primer Syl1-P (Ⓟ $^{5'}$ATT GTC ACC ATA AGC AG$^{3'}$) was a 5′-phosphorylated downstream primer whose hybridization region was from nucleotides 587 to 603 on poliovirus RNA, based on Toyoda's consensus sequence nomenclature [Toyoda et al., 1984]. Primer RB2 ($^{5'}$GTG TGA AGA GCC TAT TG$^{3'}$) was an upstream primer whose hybridization region was located at nucleotides 418 to 434.

A second PCR primer set (Lau2-P and RB1) was used to obtain a 247-250 bp PCR product. Primer Lau2-P (Ⓟ $^{5'}$GCG TTG CGC TCA GCA C$^{3'}$) was a 5′-phosphorylated upstream primer whose hybridization region was located from nucleotides 279 to 295. Primer RB1 ($^{5'}$CTT GCG CGT TAC GAC$^{3'}$) was the downstream primer with a hybridization region spanning nucleotides 516 to 530.

Sequence was determined with both primer sets, allowing 2 independent and complementary readings.

Reverse transcription. Reverse transcription was carried out either with primer Syl1-P or with primer RB1.

The following reagents were added to the viral lysate to obtain the desired final concentration: 22 to 25 U of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim), 20 U of RNase Inh, 50 mM Tris (pH = 8.3), 40 mM KCl, 1 mM DDT, 6 mM $MgCl_2$, 0.5 μM downstream primer (Syl1-P or RB1), and 1 mM each of dNTP (dATP, dCTP, dGTP and dTTP). Final reaction volume was 20 μl. cDNA transcripts were prepared by incubation for 30 min at 37°C.

PCR amplification. After reverse transcription, 80 μl of the PCR mixture was added to the whole reaction product (20 μl of DNA transcript). The final concentration of reagents was 20 mM Tris (pH = 8.3), 58 mM KCl, 2.7 mM $MgCl_2$, 0.2 mM DTT, 0.01% gelatin (Sigma; St. Quentin Fallavier, France), 0.5 μM upstream primer (Lau2-P or RB2), 0.5 μM downstream primer (Syl1-P or RB1), 5% (vol/vol) dimethyl sulfoxide (DMSO; Sigma), 0.2 mM each of dNTP, 2.5 U of Taq polymerase (Amplitaq; Perkin Elmer; St. Quentin Yvelines, France). The reaction mixture was overlaid with 2 drops of mineral oil (Sigma), and the reaction was carried out in a thermal cycler (Perkin Elmer).

After initial denaturation for 10 min at 94°C, cycling conditions were, denaturation (2 min at 94°C), primer annealing (1 min at 44°C) and strand extension (2 min at 70°C). Thirty cycles were performed, and the reaction was completed by a final extension for 5 min at 70°C. The procedure lasted 3 hr.

PCR product analysis. After extraction with chloroform/isoamyl alcohol (24 vol/1 vol), PCR products were electrophoresed in 4% agarose gel [3:1; Type XI low geling temperature agarose (Sigma): Ultrapure agarose (GIBCO-BRL; Eragny, France)] containing 0.5 μg/ml ethidium bromide. After electrophoresis (1 hr at 100 volts), DNA fragments were examined under UV light (312 nm).

Lambda exonuclease digestion. After extraction with phenol/chloroform/isoamyl alcohol (25 vol/24 vol/1 vol), the DNA was precipitated at –70°C for 1 hr with 2 volumes of absolute ethanol in the presence of 1/10 volume of 3M sodium acetate, pH = 5.5.

After washing with 70% ethanol, the dried DNA pellet was resuspended in 10 μl of Lambda exonuclease digestion buffer (67 mM glycine, 2.5 mM $MgCl_2$, pH = 9.4). The double-strand amplified product was treated with 1 U of Lambda exonuclease (GIBCO-BRL) for 20 min at 37°C [Higuchi and Ochman, 1989]. The phosphorylated strand of the amplified DNA is preferentially digested by Lambda exonuclease. The remaining single-stranded DNA was used then for direct sequencing as described below.

Direct sequencing of single-stranded DNA. The dideoxynucleotide-chain termination sequencing method [Sanger et al., 1977] was performed with Taq DNA polymerase (Amplitaq, Perkin Elmer) using Syl1-P or Lau2-P as the sequencing primer.

Partial sequencing allowed the reading of a fragment that corresponded to about 65% of the PCR product size.

The labeling and termination reactions were done at 42°C and 70°C, respectively. Electrophoresis was run at 70 watt and 2 kvolt on a 6% acrylamide gel [8M urea (Sigma)] for approximately 3 hours.

Nested PCR for type-specific amplification and partial sequencing of poliovirus genome.

Primers. Primers were selected to specifically amplify the Sabin strains and their progenitor strains for each serotype. The nucleotide sequence and primer location on poliovirus sequence are indicated below.

Lau9-P (upstream): Ⓟ $^{5'}$CTG ATG AGT CTG GAC ATC$^{3'}$ (319–336)
Lau4 (downstream): $^{5'}$GGC CAA TCA CTG GTT TGT$^{3'}$ (497–514)
Lau5-P (upstream): Ⓟ $^{5'}$CGC CAT AGG ACG TTA GA$^{3'}$ (391–407)
Lau6 (downstream): $^{5'}$AGC CAG TCA CTG GTT CGC$^{3'}$ (497–514)
Lau7-P (upstream): Ⓟ $^{5'}$GTC CCC ACT GGC GAC A$^{3'}$ (335–350)
Lau8 (downstream): $^{5'}$TGG CTG CTG GGT TGC AG$^{3'}$ (495–511)

Primers specificity was tested on reference strains (data not shown).

First amplification: RT/PCR. cDNA synthesis, PCR amplification, and PCR product analysis were carried out as described in section C-5. However, the primers Syl1-P and Lau2-P were used to obtain a larger PCR product (319–322 bp).

Second amplification: type-specific nested PCR. Nested PCR used either type 1 Sabin-related primer set (Lau9-P and Lau4), type 2 Sabin-related primer set (Lau5-P and Lau6), or type 3 Sabin-related primer set (Lau7-P and Lau8).

Lau9-P/Lau4 led to the amplification of a 192 bp fragment.
Lau5-P/Lau6 led to the amplification of a 123 bp fragment.
Lau7-P/Lau8 led to the amplification of a 176 bp fragment.

One μl of the first PCR product was used for the second amplification. The reaction conditions of the type-specific amplification were: 10 mM Tris (pH = 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatine, 0.5 μM each of type specific primers, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 5% (V/V) DMSO, and 2.5 U of Taq polymerase (Amplitaq, Perkin Elmer).

The reaction was carried out in a thermal cycler as described above; although, the annealing step was performed at 54°C for type 1-specific nested PCR.

PCR product analysis, Lambda exonuclease digestion, and partial sequencing were carried out as described in "RT/PCR and sequencing of the cloned isolates."

MAPREC test. Reversion percentage at the studied positions for the 4 cloned isolates tested with neurovirulence test on monkeys was determined by mutant analysis by PCR and restriction enzyme cleavage (MAPREC). For each poliovirus serotype, this method allowed the creation of a restriction site near the studied mutation position. PCR products obtained from reverted strains can be digested with a specific enzyme that did not digest Sabin strains PCR products, enabling the relative quantitation between Sabin and reverted genotypes [Chumakov et al., 1991].

We applied this method on one type 1 cloned isolate in order to analyse positions 480 and 525. This latter position is reported to form a base-pair with position 480 in the RNA secondary structure [Muzychenko et al., 1991] and is also related to neurovirulence [Rezapkin et al., 1994]. Determination of 480-A percentage was performed after PCR amplification with primer set (pS-1/453:pA-1/482) followed by digestion with restriction enzyme *Alu*I, as described [Lu et al., 1993]. Determination of 525-U percentage was performed after PCR amplification with primer set (pS-1/516: pA-1/527) followed by digestion with restriction enzyme *Scr*FI [Rezapkin et al., 1994].

The test also was applied on 1 type 2 and 2 type 3 cloned isolates in order to analyse positions 481 and 472, respectively. Determination of 481-G percentage was performed after PCR amplification with primer set (pS-2/465: pA-2/483) followed by digestion with the restriction enzyme *Bsp*1286I [Lu et al., 1993]. Determination of 472-C percentage was done using PCR amplification with primer set (pS-3/470: pA-3/484) and digestion with restriction enzyme *Mbo*I [Lu et al., 1993].

Statistical Analysis

Results of virologic and genetic investigations were submitted to appropriate statistical tests (e.g., analysis of variance, Kolmogorov-Smirnov test, Mantel-Haenszel test, and U-test) [Finney, 1978; Snedecor and Cochran, 1957].

RESULTS

Kinetics of Viral Excretion

Global pattern of viral excretion. Excreted viruses were isolated from 757 stool samples. Most of the stools were collected as described in the protocol, except for those of a few children who were not available at the time of the collection. Global kinetics and frequency of poliovirus excretion for the two poliovirus vaccines did not differ significantly (Kolmogorov-Smirnov test).

Pattern of viral excretion by serotype (Fig. 1).

Vero-OPV. For poliovirus type 1, excretion was extensive after the first dose as 11 of 12 children (11/12)

had virus-positive stools up to day 4. There was less virus excretion after the second (4/11) and the third doses (4/12).

Poliovirus type 2 was well excreted after the first 2 doses (10/12 and 6/11), but excretion decreased following the third dose (only 3/9 at day 61).

Poliovirus type 3 was not excreted after the first dose. The excretion began after the second and third doses, but to a lesser degree (with a maximum of 5/9) than did excretion in the PMK group. This was the only type found at day 120.

PMK-OPV. For poliovirus type 1, excretion was extensive up to day 4 (6/9) and then decreased after the second and third doses but persisted until day 120.

All children excreted poliovirus type 2 during the first month (9/9 at days 6 and 10), 4/9 after the second dose, but only 1 after the third dose.

Poliovirus type 3 excretion was limited until day 10, when 8/9 children excreted. This continued up to day 40; however, only 1 child excreted type 3 after the third dose.

When Vero-OPV and PMK-OPV were compared according to specific virus types using the Kolmogorov-Smirnov test, the difference was significant for type 1 ($P = 0.01$), not significant for type 2, and highly significant ($P < 0.01$) for type 3. Overall, the Vero-OPV group excreted type 3 poliovirus later and less frequently than the PMK-OPV group.

Frequency of Viral Isolation/Detection

In the panel of 28 "P0" stools (7 children vaccinated with Vero-OPV, 7 children vaccinated with PMK-OPV) selected at days 6 and 36, frequency of viral isolation (Method A) and frequency of viral detection by PCR (Method B) were compared (Table I). The number of viruses isolated was significantly ($P = 0.007$, analysis of variance) and consistently higher for each serotype with nested PCR (method B).

When these methods were applied to titration of the Sabin reference strains, it was noted that the sensitivity of type-specific nested PCR was tenfold higher than the sensitivity of the in vitro culture methods (data not shown). This higher sensitivity of type-specific nested PCR explains why more viral isolates could be detected with this method.

When only the method of detection by PCR in "P0" stool was considered, there was significantly more ($P = 0.014$, analysis of variance) type 2 poliovirus excreted at day 6 than at day 36, in both groups (PMK and Vero). For type 1, a similar tendency was observed, but it was not significant ($P > 0.50$, analysis of variance). For type 3, there was less excretion at day 6 in the Vero-OPV group (1/7), compared with viral excretion observed in PMK-OPV group (5/7). However, this difference was not significant ($P > 0.50$, analysis of variance).

The overall frequency of viral isolation/detection differed with the type of poliovirus. There was a tendency for poliovirus type 2 to be excreted more frequently (75%) than the other serotypes. When the pattern of viral isolation/detection frequency was observed, no significant ($P > 0.50$, analysis of variance) difference could be detected between the 2 groups, Vero-OPV and PMK-OPV.

Neutralizing Antibodies

Seroconversion (defined as a fourfold increase in poliovirus neutralizing antibodies) was observed in almost all children in both groups (Fig. 2) as early as day 60 (30 days after the second OPV dose). Some blood samples were not collected because children were unavailable. No significant difference (Mantel-Haenszel test, $P > 0.50$) could be detected between the 2 groups.

Characterization of Cloned Isolates

Phenotypic characterization by monoclonal antibody and rct40 marker test never detected wild-type virus in the 70 studied cloned isolates. It was possible to determine reversion frequency (Table II) by genotypic characterization (RT-PCR and sequencing). For type 3, frequency of reversion is identical between the two groups PMK-OPV and Vero-OPV. For type 1 and type 2 poliovirus, Vero-OPV group tends to exhibit a higher frequency of reversion. However, when a statistical analysis is applied (U-test), this difference is not significant.

Type 1 isolates were more stable than type 2 and type 3 isolates. The latter were totally reverted in both groups.

Genotypic Characterization of "P0" Stool

Twenty eight "P0" stools provided by 14 vaccinated children (7 PMK-OPV; 7 Vero-OPV) were analyzed by type-specific nested PCR and sequencing. Frequency of reversion was determined after sequencing (Table III). For 2 samples, it was necessary to dilute initial sample (tenfold) to overcome PCR inhibitions. Virus concentration by ultracentrifugation on sucrose cushion was performed for one sample to obtain reproducible results with PCR. For two type 1 isolates, sequencing was not possible because the amount of PCR product was not sufficient, even after concentration by ultracentrifugation. Although a higher frequency of reversion is observed for type 1 in Vero-OPV group, the statistical analysis (U-test) demonstrates no significant difference between PMK and Vero for all serotypes. Reversion rate was high for type 2 and type 3 and low for type 1 in both groups. Comparing these results with the frequency of reversion assessed by RT/PCR and sequencing (Table II) showed no significant difference (analysis of variance).

Neurovirulence Test and MAPREC Test

Neurovirulence testing on monkeys assessed the neurovirulence potential of 4 cloned isolates found to have reverted, based on RT/PCR and sequencing (Table IV). A MAPREC test was also performed on the same isolates to accurately determine the percentage of mutants at positions 480 and 525 (for type 1), 481 (for type 2), and 472 (for type 3). According to the monkey neurovirulence test [WHO, 1990], the reverted cloned iso-

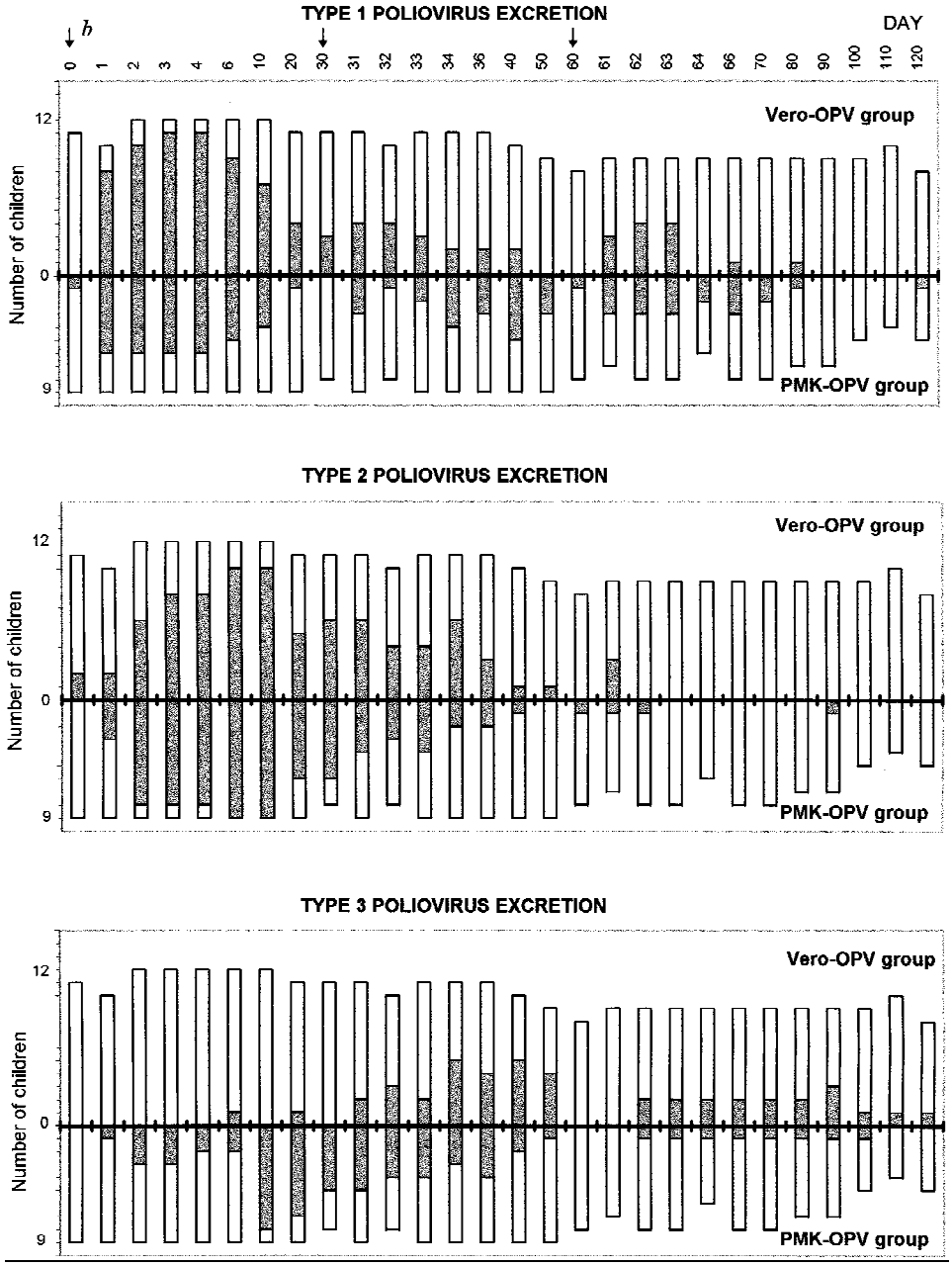

Fig. 1. Kinetics and frequency of poliovirus excretion[a] by serotype. [a]■: Children with positive viral isolation □: Children with negative viral isolation [b]arrows indicate OPV vaccination.

lates had significant higher lesion scores than in the respective WHO reference. For the MAPREC test, reversion percentages of cloned isolates were not different from 100% reverted cDNAs for position 480 (type 1), 481 (type 2), and 472 (type 3). Only 0.18% mutations were observed at position 525 for type 1 isolate. This was within the range of expected mutation in Sabin 1 original strain [Rezapkin et al., 1994]. Moreover, among the 70 sequenced cloned isolates, sequencing results from 2 type 1 cloned isolates showed an absence of mutation at position 525.

DISCUSSION

Traditionally, OPV vaccine has been produced on PMK cells, for which some concern was raised about their availability and their safety [Montagnon, 1989]. In contrast, Vero is a well-characterized cell line devoid of any contaminants [Vincent-Falquet et al., 1989]. In 1988, it was, therefore, proposed to use the Vero cell line as the cell substrate for OPV production. As such, it was necessary to demonstrate that the new cell substrate and modifications of the cell culture parameters did not adversely affect the features of the OPV vaccine. The aim of this comparative study was to assess the bioequivalence of 2 oral poliovaccines which were produced either in PMK cells or in Vero cells. Two groups of children have been studied: 1 group was vaccinated with PMK-OPV, the other with Vero-OPV. These groups of children were located at different sites

TABLE I. Frequency of Poliovirus Isolation/Detection Assessed by Two Methods

Poliovirus isolate	Type 1		Type 2		Type 3	
Methods	A[a]	B[b]	A	B	A	B
Vero-OPV (n = 14 stools)						
Day 66[c]	6/7	6/7	5/7	6/7	1/7	1/7
Day 36[c]	2/7	4/7	2/7	4/7	3/7	6/7
PMK-OPV (n = 14 stools)						
Day 6[c]	4/7	5/7	7/7	7/7	2/7	5/7
Day 36[c]	2/7	4/7	1/7	4/7	4/7	4/7
Total number of viral isolation (n = 28 stools)	14/28 (50%)	19/28 (68%)	15/28 (54%)	21/28 (75%)	10/28 (36%)	16/28 (57%)

[a]Method A: detection after in vitro culture followed by phenotypic identification.
[b]Method B: detection in "P0" stool by type-specific nested PCR.
[c]After the first dose of OPV.

to avoid cross-contamination of 1 group by excreted polioviruses from the other group.

No significant differences in neutralizing antibody levels, global kinetics, or frequency of viral excretion and reversion rate could be observed between PMK-OPV and Vero-OPV. In addition, since its introduction in 1989, several hundred million doses of Vero-OPV have been distributed worldwide with no significant increase in vaccination-related adverse side effects. This constitutes evidence in favor of the use of Vero cells for the large-scale production of OPV, even though some differences are observed for viral excretion by serotype, for type 1 and mainly for type 3 poliovirus. In this last case, the Vero-OPV group excreted type 3 polioviruses later and less frequently than the PMK-OPV group. However these differences, observed for kinetics of excretion, have no incidence on reversion frequency which is similar in both groups.

The intention of this study was to improve the understanding of the natural events occurring in vivo following vaccination of healthy people. The nucleic-acid based methods designed for the purpose of this study have highlighted the limits of classic virological methods in the characterization of viral strains. For example, classic phenotyping methods do not regularly correlate with the genetic analysis. Although, RT/PCR and sequencing method have proved to be suitable for discriminating reverted strains from Sabin strains, this procedure was time consuming as viral isolation and cloning on cell culture was necessary. Since reversion patterns have been shown to be strongly influenced by culture, particularly at temperatures exceeding 34°C [Chumakov et al., 1992; Rezapkin et al., 1994; Taffs et al., 1995], a new PCR-based method was designed to detect poliovirus directly in stool specimens and overcome these selection parameters.

We selected a type-specific, nested PCR which proved to be 10 times more sensitive than viral isolation on cell culture. Nested PCR increased sensitivity and specificity of the viral detection [Leparc et al., 1993]. With this type-specific nested PCR method, viral detection was performed within 12 hr and further sequencing could be done.

Applied to 28 stools specimens, type-specific, nested PCR proved to be more efficient than cell culture. PCR inhibition problems that were encountered in 2 cases were easily overcome by diluting the original samples tenfold.

When results from both genetic methods (RT-PCR and sequencing vs. nested PCR and sequencing) were analysed, reversion frequency was not significantly different. Since the RT/PCR and sequencing method (which requires preliminary cell culture) and the nested PCR and sequencing method did not give significantly different results, the successive passages on cells did not seem to influence the reversion rate at temperatures above 34°C, as has been previously shown [Chumakov et al., 1992; Rezapkin et al., 1994; Taffs et al., 1995]. It is probably because the passage number was lower than that in earlier studies. Reversion for each poliovirus type in PMK-OPV vaccinees was consistent with previously published results [Minor and Dunn, 1988; Dunn et al., 1990], and the high frequency of reversion observed for type 2 and type 3 (with PMK-OPV and Vero-OPV) suggested that the attenuated Sabin genotype is, in fact, highly unstable.

When the 4 cloned isolates were analyzed by the neurovirulence test [WHO, 1990], the observed lesion scores indicated the neurovirulent features of these isolates, which is in accordance with the reported relationship between 5′-noncoding region point mutations and neurovirulence [Cann et al., 1984; Evans et al., 1985; Kawamura et al., 1989; Westrop et al., 1989; Macadam et al., 1993]. However, other mutations on the genome or intertypic recombinations could also be involved in this neurovirulence enhancement.

MAPREC test showed that the 4 tested isolates reverted completely at positions 480 for type 1, 481 for type 2, and 472 for type 3. These results are consistent with the observation that reversion percentages in a viral suspension correlate strictly with mean lesion scores in the monkey test [Chumakov et al., 1992; Rezapkin et al., 1994; Taffs et al., 1995]. The low percentage of mutations obtained at position 525 suggested that mutation of poliovirus type 1 in stools occurred in vivo preferentially at position 480. The increase in mean lesion score for tested type 1 cloned isolate was therefore directly related to reversion at position 480.

Thus the MAPREC test and the neurovirulence test on monkeys corroborated that there is a direct correlation between mutations 480 in type 1, 481 in type 2,

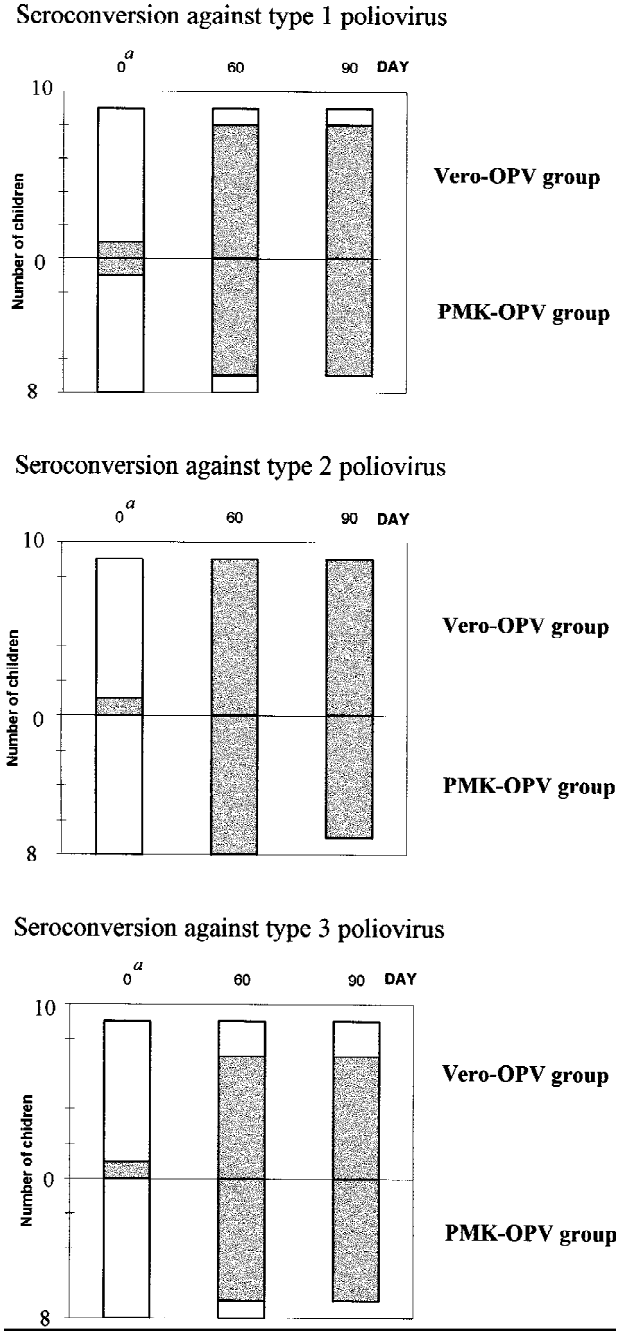

Fig. 2. Kinetics of seroconversion by serotype. ■: Children with neutralizing antibodies □: Children without neutralizing antibodies. [a]Some children are exhibiting low level of neutralizing antibodies prior to vaccination.

and 472 in type 3, and an elevated neurovirulence score. This confirms the usefulness of the MAPREC test [Chumakov et al., 1991, 1992] for the regular control of monovalent OPV production.

The observation that these same positions undergo reversion in vaccine-associated poliomyelitis cases (e.g., in type 1 isolates [Otelea et al., 1993], in type 2 isolates [Pollard et al., 1989; Equestre et al., 1991], and in type 3 isolates [Cann et al., 1984; Evans et al., 1985]) raises the question of safety of OPV for first and second

TABLE II. Frequency of Poliovirus Reversion* on Cloned Isolates Determined After RT/PCR and Sequencing

Cloned isolates (n = 70)			Vero-OPV			PMK-OPV	
Type 1	Day 6 Day 36	2/12 0/1	2/13	15%	0/6 0/0	0/6	0%
Type 2	Day 6 Day 36	14/18 2/4	16/22	73%	9/15 2/2	11/17	65%
Type 3	Day 6 Day 36	0/0 5/5	5/5	100%	6/6 1/1	7/7	100%

*According to genotypic characterization of studied positions for type 1 (position 480), type 2 (position 481), and type 3 (position 472) poliovirus.

vaccine administration, as most vaccine-associated paralytic poliomyelitis (VAPP) cases occur after these doses [Nkowane et al., 1987; Strebel et al., 1992; Prevots et al., 1994]. In the United States, the VAPP risk between 1973 and 1984 has been estimated at 1 case per 520,000 first doses distributed [Nkowane et al., 1987].

Furthermore, in a recent, large-scale epidemiological study from Brazil covering 1626 patients with sudden onset of acute flaccid paralysis [De Filippis et al., 1994], evidence indicated that all paralytic cases reported as poliomyelitis cases between January 1990 and September 1993 were related not to wild-type polioviruses, but to vaccinal polioviruses. In Brazil, the wide use of OPV has proved efficient in stopping the wild-type poliovirus related poliomyelitis, but this study highlights the problem of vaccine-related poliovirus circulation and the resulting vaccine-associated paralytic poliomyelitis.

In France, an epidemiological survey between 1966 and 1980 [Netter et al., 1982] reported that there were 29 VAPP cases for 62 million OPV doses distributed. This led to a change in the vaccination policy, which was implemented in 1982. The new French vaccination schedule consists of 3 injections of enhanced-potency inactivated poliovaccine (E-IPV) at 2, 3, and 4 months followed by further doses either of OPV or E-IPV between 12 and 15 months, 5 and 6 years, and 11 and 13 years, followed by a dose every 10 years. The last reported French VAPP case was in 1983. The most recent clinical poliomyelitis cases in France occurred in 1989 in 2 unvaccinated adults. Since 1990, surveys of sewage and of day care centers have revealed no wild-type poliovirus. Since 1993, no isolation of wild polioviruses has been reported in hospitals. Furthermore, poliovirus circulation has decreased markedly in France, and only Sabin-related polioviruses continue to circulate [Roure et al., 1991; Roure, 1991]. This provides evidence of the vaccination schedule efficiency in France, where distributed OPV doses represent now only 1.2% of the total number of poliovaccine doses distributed in 1994.

A sequential vaccine scheme (3 doses of IPV followed by 3 doses of OPV) has been successfully applied since 1968 in Denmark [Von Magnus and Petersen, 1984]. A combined vaccine scheme (IPV/OPV) has been success-

TABLE III. Frequency of Poliovirus Reversion* on "P0" Stool Determined After Type-Specific Nested PCR and Sequencing

Type		Vero-OPV		PMK-OPV[a]	
1	Sabin	6/10		6/7	
	Mix reverted/Sabin	3/10	4/10 (40%)[b]	1/7	1/7 (14%)[b]
	Reverted	1/10		0/7	
2	Sabin	2/10		2/11	
	Mix reverted/Sabin	3/10	8/10 (80%)[b]	3/11	9/11 (82%)[b]
	Reverted	5/10		6/11	
3	Sabin	1/7		0/9	
	Mix reverted/Sabin	3/7	6/7 (86%)[b]	1/9	9/9 (100%)[b]
	Reverted	3/7		8/9	

*According to genotypic characterization of studied positions:
For type 1 (position 480) = G → Sabin
G + A → Mix reverted/Sabin
A → Reverted
For type 2 (position 481) = A → Sabin
A + G → Mix reverted/Sabin
G → Reverted
For type 3 (position 472) = T → Sabin
T + C → Mix reverted/Sabin
C → Reverted
[a]Sequencing was not possible with two type 1 isolates in PMK-OPV group.
[b]Frequency of reversion.

TABLE IV. Results of Monkey Neurovirulence Test and MAPREC Test Performed on Four Cloned Isolates

Poliovirus type	Cloned isolates (test sample)	Mean lesion score obtained with neurovirulence test on monkeys		% of reversion obtained with MAPREC test	
		Test sample	WHO reference	Test sample	100% reverted cDNA reference
Type 1	H78CB	1.038 ± 0.184[a] (12/12)[b]	0.565 ± 0.192[a] (11/12)[b]	96.27% at position 480 0.18% at position 525	96.86% at position 480 91.72% at position 525
Type 2	H67EA	0.469 ± 0.108[a] (12/12)[b]	0.280 ± 0.108[a] (12/12)[b]	95.65% at position 481	95.19% at position 481
Type 3	H275B	0.822 ± 0.236[a] (19/20)[b]	0.342 ± 0.274[a] (14/20)[b]	96.16% at position 472	94.59% at position 472
	H287A	0.976 ± 0.230[a] (20/20)[b]	0.342 ± 0.274[a] (14/20)[b]	95.89% at position 472	94.59% at position 472

[a]95% confidence interval of the mean lesion score.
[b]Number of positive monkeys/number of test monkeys. A "positive" monkey is one in which neuronal lesions characteristic of poliovirus are seen in the central nervous system according to the WHO criteria [WHO, 1990].

fully used in the West Bank and Gaza since 1978 and was later on adopted in Israel [Tulchinsky et al., 1994].

Moreover, a study of immunization schedule using a combination of E-IPV and OPV has shown that the incorporation of at least 1 dose of E-IPV at the start of vaccine schedule tends to increase systemic as well as local antibody production [Faden et al., 1990].

On the basis of the postulate that a vaccine should not transmit the disease that it is intended to prevent and because some OPV immunogenicity failures have been reported in developing countries [Patriarca et al., 1991], a sequential scheme of vaccination (E-IPV/OPV) seems to be the best way to achieve poliomyelitis eradication. Even though prior immunization with IPV will not prevent fecal excretion of poliovirus revertants if OPV is later administered [Abraham et al., 1993], it will nevertheless protect vaccinees from VAPP risk.

Fecal shedding of neurovirulent revertants suggests also that high vaccine coverage is required to protect contacts from this residual iatrogenic hazard.

ACKNOWLEDGMENTS

We are grateful to all the physicians who have conducted the clinical study: Drs. Betend, Chalier, Dieu, Gillet, and Oudot. We thank Dr. R. Sodoyer (P.M.sv., research department), for providing oligonucleotide primers; Dr. K. Chumakov (FDA-CBER, Bethesda, MD), for providing primers and cDNAs used with MAPREC method; Dr. S. Plotkin (P.M.sv., medical department) and Dr. H. Kopecka (Institut Pasteur, Paris), for critical analysis of this manuscript; Dr. M. Fletcher, for editorial assistance (P.M.sv., medical department); C. Michel, for technical assistance; and C. Boulot, for typing the manuscript. L. Mallet is a fellow of the Fondation Marcel Mérieux.

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